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Improved Tuberculosis DNA Vaccines by Formulation in Cationic Lipids

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Mice were vaccinated with plasmid DNA (pDNA) encoding antigen 85A (Ag85A), Ag85B, or PstS-3 from *Mycobacterium tuberculosis* either in saline or formulated for intramuscular injections in VC1052:DPyPE (aminopropyl-dimethyl-myristoleyloxy-propanaminium bromide–diphytanoylphosphatidyl-ethanolamine) (Vaxfectin; Vical, Inc., San Diego, Calif.) or for intranasal instillations in GAP-DLRIE:DOPE (aminopropyl-dimethyl-*bis*-dodecyloxy-propanaminium bromide–dioleoylphosphatidyl-ethanolamine). These two novel cationic and neutral colipid formulations were previously reported to be effective adjuvants for pDNA-induced antibody responses. The levels of Ag85-specific total immunoglobulin G (IgG) and IgG isotypes were all increased 3- to 10-fold by formulation of pDNA in Vaxfectin. The level of production of splenic T-cell-derived Th1-type cytokines (interleukin-2 and gamma interferon) in response to purified Ag85 and to synthetic peptides spanning the entire Ag85A protein was also significantly higher in animals vaccinated with pDNA formulated in Vaxfectin. Cytolytic T-lymphocyte responses generated by pDNA encoding phosphate-binding protein PstS-3 in Vaxfectin were better sustained over time than were those generated by PstS-3 DNA in saline. Intranasal immunization with Ag85A DNA in saline was completely ineffective, whereas administration in GAP-DLRIE:DOPE induced a positive Th1-type cytokine response; however, the extent of the latter response was clearly lower than that obtained following intramuscular immunization with the same DNA dose. Combined intramuscular and intranasal administrations in cationic lipids resulted in stronger immune responses in the spleen and, more importantly, in the lungs as well. Finally, formulation in Vaxfectin increased the protective efficacy of the Ag85B DNA vaccine, as measured by reduced relative light unit counts and CFU counts in the spleen and lungs from mice challenged with bioluminescent *M. tuberculosis* H37Rv. These results may be of importance for future clinical use of DNA vaccines in humans.

Tuberculosis (TB) remains a major health problem affecting millions of people worldwide. The only TB vaccine currently available is an attenuated strain of *Mycobacterium bovis* termed bacillus Calmette-Guérin (BCG). The efficacy of BCG remains controversial, particularly against pulmonary TB in young adults, and the development of an improved vaccine is urgently needed to counter the global threat of this disease (23, 24).

Extracellular and surface-exposed cell wall proteins from the pathogen are thought to be important for the elicitation of protective immune responses against TB. A major fraction of the secreted proteins in *M. tuberculosis* and BCG culture filtrates is formed by the antigen 85 (Ag85) complex (43), a 30- to 32-kDa family of three proteins (Ag85A, Ag85B, and Ag85C) which all possess enzymatic mycoyltransferase enzyme activity involved in the attachment of mycolic acids to the arabinogalactan of the cell wall and in the biogenesis of cord factor (33). The Ag85 complex is a promising vaccine candidate, as it sensitizes the immune system for strong T-cell proliferative responses and gamma interferon (IFN-γ) production in most healthy individuals infected with *M. tuberculosis* or *M. leprae* (25) and in BCG-vaccinated mice (18) but not in TB or lepromatous leprosy patients (21, 26). It has been reported that immunization with naked plasmid DNA (pDNA) encoding Ag85A and Ag85B can stimulate strong humoral and cell-mediated immune responses and confer significant protection to C57BL/6 mice challenged by the aerosol or intravenous route with live *M. tuberculosis* H37Rv (1, 19, 22). Recently, priming with Ag85B DNA was shown to augment the protective efficacy of *M. bovis* BCG (10), and recombinant BCG overexpressing Ag85B was found to have increased immunogenicity and efficacy in guinea pigs (16). Finally, a fusion protein consisting of Ag85B and ESAT-6 is a very promising protein-subunit vaccine candidate for TB (41).

Another promising TB DNA vaccine consists of DNA encoding the 40-kDa protein PstS-3 (38). PstS-3, PstS-1 (also called the 38-kDa antigen), and PstS-2 are surface-exposed lipoproteins that are putative mycobacterial phosphate transport proteins, homologous to phosphate-binding protein PstS of *Escherichia coli* (2, 27).

Although the immunogenicity of DNA vaccines in humans is promising, increasing the potency of DNA vaccines is a clear necessity (40). Increased pDNA-induced antibody responses can be obtained, among others, by complexation with conventional adjuvants, such as monophosphoryl lipid A (34), alum (40), and QS-21 saponin (35). Priming with DNA followed by boosting with either purified protein (37) or recombinant modified vaccinia virus Ankara (29) was also shown to increase the immunogenicity and protective efficacy of DNA vaccines consisting of DNA encoding Ag85A. Here we report on an approach for improving TB DNA vaccines by formulation in two novel cationic and neutral colipid formulations, GAP-DLRIE:DOPE (aminopropyl-dimethyl-*bis*-dodecyloxy-propanaminium bromide–dioleoylphosphatidyl-ethanolamine) and VC1052:DPyPE (aminopropyl-dimethyl-myristoleyloxy-propanaminium bromide–diphytanoylphosphatidyl-ethanolamine), the

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latter also called Vaxfectin (Vical, Inc., San Diego, Calif.). Both formulations were previously demonstrated to enhance antibody responses to pDNA given by the intranasal and intramuscular routes, respectively (13, 32; L. Sukhu, M. Wloch, M. Sawdey, C. Wheeler, and M. Manthorpe, Abstr. 2nd Annu. Meet. Am. Soc. Gene Ther., abstr. 530, 1999). We show that these lipids can be used as adjuvants for DNA-based vaccination with Ag85 and PstS-3 from *M. tuberculosis*, resulting in significantly increased antibody titers and levels of Th1-type cytokine production in the spleen and lungs and more sustained cytolytic T-lymphocyte (CTL) responses as well as increased protective efficacy against an intravenous challenge with bioluminescent *M. tuberculosis* H37Rv.

MATERIALS AND METHODS

Plasmid construction. pDNA encoding Ag85A, Ag85B, and PstS-3 from *M. tuberculosis* was prepared as described before (19, 28, 38).

Mice. C.D2 mice (BALB/c background, *Bcg*^{r} allele) and C57BL/10 (B10) mice were bred in the animal facilities of the Pasteur Institute of Brussels from breeding pairs originally obtained from E. Skamene (McGill University, Montreal, Quebec, Canada) and R. ten Berg (Netherlands Cancer Institute), respectively. C57Bl/6 (B6) mice were obtained from Bantin and Kingman (Grimston, United Kingdom). Only female mice, 6 to 8 weeks old at the start of vaccination, were used.

DNA immunizations. Mice were anesthetized by intraperitoneal injections of ketamine-xylarine. For intramuscular immunizations, mice were injected in both quadriceps or tibialis anterior muscles with two 50-μl volumes (total dose, 50 μg) of empty vector (control DNA) or plasmid vector containing Ag85A, Ag85B, or PstS-3 DNA in saline or formulated in Vaxfectin at a pDNA/cationic lipid molar ratio of 2:1. For intranasal immunizations, two 10-μl volumes of pDNA (total dose, 20 μg of DNA) in saline or formulated in GAP-DLRIE:DOPE (molar ratio, 4:1) were deposited in the left and right nares with a resting time of 60 s between the two instillations. Mice were immunized by the intramuscular, intranasal, or combined routes three or four times at 3-week intervals (as detailed further below).

For BCG vaccination, mice were injected intravenously with 0.1 mg (about 5×10^5 CFU) of *M. bovis* BCG (strain GL2), freshly prepared from surface-grown pellicles on synthetic Sauton medium.

ELISA. Sera from pDNA-immunized mice were collected by retro-orbital bleeding 3 weeks after the last immunization. For collection of bronchoalveolar fluid, mice were sacrificed by cervical dislocation and lungs were gently rinsed with 1 ml of phosphate-buffered saline injected with an 18-gauge needle-syringe through a narrow split in the trachea. Levels of total anti-Ag85 immunoglobulin antibodies were determined by an enzyme-linked immunosorbent assay (ELISA) with sera from individual mice (three to five per group). The serum titer was converted to antibody concentration (nanograms per milliliter) by comparison with a standard monoclonal antibody with known potency, and the mean antibody concentration was calculated from at least three points of the linear portion of the titration curve. Concentrations were converted to $\log_{10}$ values. For immunoglobulin isotype analysis, equal volumes of sera were pooled per group and examined with peroxidase-labeled rat anti-mouse IgG1, IgG2a, IgG2b, and IgA (Experimental Immunology Unit, Université Catholique de Louvain, Brussels, Belgium). Immunoglobulin isotype titers were converted to arbitrary units by comparison with a standard serum pool from Ag85 DNA-immunized mice, arbitrarily assigned a titer of 1,000 for all isotypes.

Antigens. Native 32-kDa Ag85A and 30-kDa Ag85B were purified from 2-week-old culture filtrates of *M. bovis* BCG (strain GL2) grown as a surface pellicle on synthetic Sauton medium by sequential chromatography on phenyl-Sepharose, DEAE-Sephacel, and Sephadex G-75 (4). Synthetic 20-mer peptides (overlapping by 10 amino acids) covering the entire mature Ag85A sequence were synthesized as described before (20). *M. tuberculosis* culture filtrates were prepared from 2-week-old cultures of *M. tuberculosis* H37Rv grown as a surface pellicle on Sauton medium and concentrated by 70% $(NH_4)_2SO_4$ precipitation (18).

Cytokine production. Vaccinated mice were sacrificed 3 weeks after the last immunization, and spleens and lungs were removed aseptically. Organs were homogenized by gentle disruption in a loosely fitting Dounce homogenizer, and lung cell suspensions were passed through a nylon-wool column to eliminate debris. Spleen cells from three or four mice per group were tested individually, whereas lung cells were pooled for each group. Cells were tested at 4×10^6 white blood cells/ml for cytokine production in response to purified Ag85A or Ag85B (5 μg/ml), culture filtrates from *M. tuberculosis* (25 μg/ml), or synthetic Ag85A peptides (10 μg/ml). Supernatants were harvested after 24 h (interleukin-2 [IL-2]) and 72 h (IFN-γ), when peak values of the respective cytokines can be measured. Supernatants from at least three separate wells were pooled and stored frozen at −20°C until assayed.

IL-2 assay. IL-2 activity was measured by using a bioassay with IL-2-dependent CTLL-2 cells as described before (18). Each sample was tested in duplicate. IL-2 levels were expressed as mean counts per minute of incorporated [^{3}H]thymidine. The standard deviation was below 10%. In this assay, a standard preparation of IL-2 at 600 pg/ml corresponded to about 15,000 cpm, and the detection limit was 30 pg/ml.

IFN-γ assay. IFN-γ activity was quantified by a sandwich ELISA with coating antibody R4-6A2 and biotinylated detection antibody XMG1.2 (both from Pharmingen). The sensitivity of the ELISA was 10 pg/ml.

Cytolytic assay. PstS-3-specific cytotoxic T-cell activity was determined with B6 mice according to a protocol previously described for Ag85A in BALB/c mice (6). Briefly, splenic lymphocytes from B6 mice vaccinated with PstS-3 DNA 1 or 5 months previously were stimulated for 1 week with D^b-restricted peptide SGVGNDLVL (amino acids 291 to 299 of the mature PstS-3 sequence), purified on a Ficoll gradient, and tested for cytolytic activity in a standard 4-h ^{51}Cr release assay; this assay was done with 10^4 ^{51}Cr-labeled RMA cells as targets (5), unpulsed or pulsed with the same peptide (5 μg/ml), at various effector/target ratios. Spontaneous or total release samples were prepared by adding targets to wells containing medium only or medium plus 2 M H_2SO_4, respectively. After 4 h of incubation at 37°C, the plates were centrifuged and 150 μl of supernatant was collected and counted in a gamma counter (LKB). Data were expressed as percent specific lysis. Spontaneous release was generally 10 to 15% of total release.

Intravenous *M. tuberculosis* H37Rv challenge. B10 mice were vaccinated intramuscularly with control DNA (empty vector V1J.ns) or Ag85B DNA in saline or in Vaxfectin or intravenously with *M. bovis* BCG. Mice were rested for 6 weeks after the third immunization and then challenged intravenously in a lateral tail vein with 10^6 CFU of luminescent, recombinant luciferase reporter *M. tuberculosis* H37Rv (36). Mice were sacrificed 14, 28, and 56 days after challenge, and the number of CFU in the lungs was enumerated by plating on 7H11 Middlebrook agar (37). The number of bioluminescent organisms (determined as relative light units [RLU]) in lung and spleen homogenates was also determined by using a bioluminescence assay with a Turner Design 20/20 luminometer and 1% *n*-decyl-aldehyde in ethanol as a substrate (36). It was shown previously that RLU counting is an easy and reliable alternative for labor-intensive CFU enumeration (37). For statistical analysis (Student's *t* test), CFU and milli-RLU (mRLU) values were converted to $\log_{10}$ values per organ per mouse; mean and standard deviation $\log_{10}$ CFU or mRLU values were calculated for each experimental group, which consisted of four to seven animals tested individually (see Table 5).

RESULTS

Formulation in Vaxfectin increases specific antibody production in Ag85 DNA-vaccinated mice. As shown in Table 1, Ag85A-specific antibody titers were more than 10-fold higher in sera from C.D2 mice vaccinated intramuscularly with Ag85A DNA formulated in Vaxfectin than in mice vaccinated with the same DNA in saline. Antibody levels in B10 mice vaccinated with Ag85B DNA in saline were about 10-fold higher than those in C.D2 mice, and Vaxfectin further increased this response, albeit more modestly than in C.D2 mice. Finally, B6 mice vaccinated with a combined intramuscular and intranasal Ag85A DNA immunization regimen also showed higher antibody responses following vaccination with DNA in cationic lipids than following vaccination with DNA in saline. Vaxfectin had an adjuvant effect on antibodies of the IgG1, IgG2a, and IgG2b isotypes. B6 mice immunized by the combined intramuscular and intranasal routes were also analyzed for mucosal antibodies in bronchoalveolar fluid, but no Ag85-specific response of either the IgG or the IgA isotype could be

TABLE 1. Increased antibody production in C.D2, B10, and B6 mice vaccinated with Ag85A and Ag85B DNA formulated in cationic lipids

Mouse strain[a]	Vaccine	Total IgG[b] (*n*)	IgG1[c]	IgG2a[c]	IgG2b[c]
C.D2	Ag85A DNA-saline	3.63 ± 0.20 (4)	575	2,110	291
	Ag85A-Vaxfectin	4.88 ± 0.27 (4)[d]	2,785	21,912	2,971
B10	Ag85B DNA-saline	4.72 ± 0.33 (5)	12,285	2,005	1,542
	Ag85B DNA-Vaxfectin	5.35 ± 0.25 (5)[e]	75,563	5,940	6,076
B6	Control DNA-saline	3.02 ± 0.06 (3)	116	115	189
	Control DNA-lipids	2.87 ± 0.21 (4)	70	147	262
	Ag85A DNA-saline	4.32 ± 0.04 (3)	37,820	4,523	3,511
	Ag85A DNA-lipids	4.87 ± 0.20 (3)[e]	165,270	6,016	11,848

[a] C.D2 mice were vaccinated with DNA encoding a mature form of Ag85, and B10 and B6 mice were vaccinated with DNA encoding a secreted form (signal sequence of human tissue plasminogen activator) of Ag85. For C.D2 and B10 mice, DNA was administered three times by the intramuscular route; for B6 mice, DNA was administered three times by the intramuscular route, in combination with two intranasal immunizations at the second and third time points.

[b] Ag85-specific IgG levels expressed as $\log_{10}$ nanograms per milliliter (mean ± standard deviation); *n*, number of mice.

[c] Expressed as arbitrary units per milliliter, calculated from a serum pool.

[d] For a comparison with titers in mice vaccinated with DNA in saline, the *P* value was <0.005.

[e] For a comparison with titers in mice vaccinated with DNA in saline, the *P* value was <0.01.

observed. Ag85-specific IgA was also undetectable in the sera of these mice.

Formulation in Vaxfectin increases specific spleen cell IL-2 and IFN-γ production. IL-2 and IFN-γ production in response to purified native Ag85 and to culture filtrates from *M. tuberculosis* was increased by the Vaxfectin formulation two- to fourfold in cultures of spleen cells from C.D2 and B10 mice immunized with DNA encoding Ag85A or Ag85B (Table 2). IL-2 and IFN-γ production in response to synthetic 20-mer peptides spanning the mature Ag85A sequence was also two-to fourfold higher in C.D2 mice vaccinated with Ag85A DNA complexed in Vaxfectin than in mice immunized with Ag85A DNA in saline (Fig. 1). Interestingly, Vaxfectin seemed to increase preferentially $CD4^+$-T-cell-mediated IFN-γ responses, whereas IFN-γ production in response to the previously defined major histocompatibility complex (MHC) class I-restricted peptides p7 and p15 (6) appeared not to be influenced by Vaxfectin.

Formulation in GAP-DLRIE:DOPE improves the immunogenicity of intranasally administered pDNA encoding Ag85A. As *M. tuberculosis* is a lung pathogen, vaccination protocols inducing local pulmonary immune responses may be important for effective control of this intracellular bacterium. We therefore examined the immunogenicity of pDNA administered by the intranasal route. The instillation of pDNA encoding Ag85A in saline was completely ineffective, probably because of degradation of the DNA by mucosal nucleases (data not shown). Intranasal immunization with pDNA complexed in GAP-DLRIE:DOPE was capable of inducing a weak splenic Th1-type immune response that was, however, much lower than the response induced by intramuscular immunization with the same dose of DNA in saline (Table 3). No local Th1-type immune response could be detected at the lung level with either intramuscular or intranasal immunization. Intranasal immunization either with saline or with GAP-DLRIE:DOPE failed to induce any detectable Ag85-specific serum immunoglobulin antibodies (data not shown).

Combined intramuscular and intranasal pDNA immunization in cationic lipids induces a local Th1-type immune response in the lungs. As shown in Table 4, a combined immunization protocol of an intramuscular injection of pDNA encoding Ag85A in Vaxfectin followed by two combined intramuscular (in Vaxfectin) and intranasal (in GAP-DLRIE:DOPE) instillations of the same pDNA resulted in an increased spleen Th1-type immune response compared to that resulting from the same immunization protocol with saline. Importantly, a local, albeit weak, Th1-type immune response could also be detected in lung cell suspensions stimulated with

TABLE 2. Increased IL-2 and IFN-γ production in C.D2 and B10 mice vaccinated with DNA encoding Ag85A and Ag85B in Vaxfectin

Mouse strain	Vaccine	Level of the following cytokine with the indicated stimulation:			
		IL-2[a]		IFN-γ[b]	
		CF	Ag85	CF	Ag85
C.D2	85A DNA-saline	9,929 ± 1,595	12,563 ± 1,517	2,544 ± 663	5,002 ± 1,319
	85A DNA-Vaxfectin	21,436 ± 4,799[c]	20,422 ± 2,134[d]	8,127 ± 1,544[c]	15,830 ± 2,446[c]
B10	85B DNA-saline	5,976 ± 430	7,155 ± 1,273	10,697 ± 1,787	17,459 ± 2,654
	85B DNA-Vaxfectin	18,076 ± 1,283[c]	20,722 ± 1,434[c]	51,963 ± 6,958[c]	42,859 ± 5,268[c]

[a] Levels (counts per minute) in spleen cell cultures stimulated for 24 h with *M. tuberculosis* culture filtrate (CF) or purified Ag85 3 weeks after the third immunization. Results are expressed as means ± standard deviations for three mice tested individually.

[b] Levels (picograms per milliliter) in spleen cell cultures stimulated for 72 h with *M. tuberculosis* CF or purified Ag85 3 weeks after the third immunization. Results are expressed as means ± standard deviations for three mice tested individually.

[c] For a comparison with results obtained with saline, the *P* value was <0.05.

[d] For a comparison with results obtained with saline, the *P* value was <0.01.

[e] For a comparison with results obtained with saline, the *P* value was <0.005.

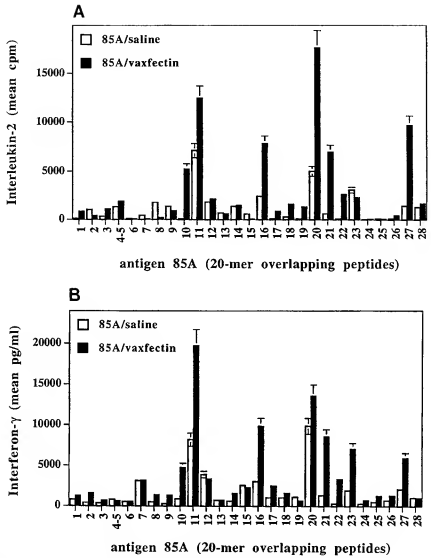

FIG. 1. IL-2 (A) and IFN-γ (B) production in cultures of spleen cells from C.D2 mice vaccinated with Ag85A DNA in saline or Vaxfectin and restimulated in vitro with synthetic 20-mer overlapping peptides spanning the entire mature Ag85A sequence. Data are means and standard deviations.

Ag85A or *H-2*^{b} immunodominant peptide p27 spanning amino acids 261 to 280 (37). The same immunization protocol with saline did not induce any local immune response in the lungs. The combined intramuscular and intranasal protocol with cationic lipids also induced a weak Ag85-specific IFN-γ response in the draining mediastinal and cervical lymph nodes (data not shown).

Formulation in Vaxfectin better sustains CTLs in spleen cells from B6 mice vaccinated with PstS-3 DNA. It was previously shown that D^b- and K^k-restricted epitopes are absent on Ag85A (9) (and Ag85B; data not shown); therefore, it is impossible to examine CTL responses to Ag85 in B6 and B10 mice. However, as we recently identified a D^b-restricted peptide on the 40-kDa PstS-3 lipoprotein, we were able to analyze the effect of the Vaxfectin formulation on CTL responses in B6 mice following vaccination with DNA encoding this phosphate-binding protein. As demonstrated in Fig. 2A, CTL activity against the D^b-restricted PstS-3 epitope was not affected

TABLE 3. Comparative immunogenicity of Ag85A DNA administered by the intramuscular route (in saline) or by the intranasal route (in GAP-DLRIE:DOPE)

Cytokine	Organ	Level after the following route of administration:	
		Intramuscular[a]	Intranasal[b]
IL-2 (cpm)[c]	Spleen	27,577 ± 11,980	6,137 ± 4,057
	Lungs	229 ± 89	255 ± 4
IFN-γ (pg/ml)[d]	Spleen	6,167 ± 2,183	1,100 ± 623
	Lungs	<5	<5

[a] Four doses of 20 μg of Ag85A DNA administered in saline intramuscularly at 3-week intervals. Spleens were from four B6 mice tested individually; lung cells were pooled 3 weeks after the last immunization.

[b] Four doses of 20 μg of Ag85A DNA administered in GAP-DLRIE:DOPE intranasally at 3-week intervals. Spleens were from four mice tested individually 3 weeks after the last immunization. Lungs from the same four mice were pooled.

[c] Mean levels measured in 24-h culture supernatants of cells stimulated with purified Ag85 protein.

[d] Mean levels measured in 72-h culture supernatants of cells stimulated with purified Ag85 protein.

TABLE 4. Combined intramuscular and intranasal DNA administration in cationic lipids elicits a stronger Th1-type immune response in spleen and lungs than the same DNA immunization in saline[a]

Organ	Vaccine	Level of the following cytokine with the indicated stimulation:					
		IL-2 (mean cpm ± SD)			IFN-γ (mean pg/ml)		
		Control	Ag85	Amino acids 261–280	Control	Ag85	Amino acids 261–280
Spleen	Control DNA-saline	1,014 ± 34	1,394 ± 407	1,043 ± 139	ND	ND	ND
	Control DNA-lipids	1,151 ± 271	1,638 ± 154	1,222 ± 139	ND	ND	ND
	Ag85A DNA-saline	849 ± 250	7,843 ± 4,628	5,080 ± 3,596	ND	ND	ND
	Ag85A DNA-lipids	1,836 ± 504	26,441 ± 4,843	20,174 ± 5,139	ND	ND	ND
Lungs	Control DNA-saline	527 ± 2	882 ± 311	712 ± 174	0	2	3
	Control DNA-lipids	756 ± 38	1,330 ± 208	951 ± 3	2	0	0
	Ag85A DNA-saline	408 ± 37	808 ± 167	556 ± 26	3	2	5
	Ag85A DNA-lipids	408 ± 30	1,998 ± 164	1,349 ± 27	0	71	46

[a] The first immunization was 50 μg of DNA intramuscularly in saline or in Vaxfectin; the second and third immunizations were 50 μg of DNA intramuscularly in saline or in Vaxfectin combined with 20 μg of DNA intranasally in saline or in GAP-DLRIE:DOPE, respectively. ND, not done.

by the Vaxfectin formulation when spleen cells were analyzed at the peak time point of 1 month after the third PstS-3 DNA injection. However, CTL activity measured 5 months after DNA vaccination was clearly more sustained in B6 mice vaccinated with PstS-3 DNA in Vaxfectin (Fig. 2B). Confirming previous results obtained with BALB/c mice vaccinated with pDNA encoding the nucleoprotein from influenza virus (13), CTL responses in C.D2 mice vaccinated with Ag85A were not affected by Vaxfectin 3 weeks after the third DNA immunization (data not shown).

Formulation in Vaxfectin improves the protective efficacy of a DNA vaccine consisting of DNA encoding Ag85B. B10 mice were rested for 6 weeks after vaccination with Ag85B DNA in saline or Vaxfectin and challenged intravenously with 10^6 CFU of recombinant luciferase reporter *M. tuberculosis* H37Rv. Throughout the 2-month challenge period, mice vaccinated with Ag85B DNA in Vaxfectin showed a highly significant decrease in RLU in the lungs compared to mice vaccinated with empty vector (Table 5). These results were confirmed by plating lung suspensions on 7H11 Middlebrook agar and enumerating actual CFU numbers (Fig. 3). Complexation in Vaxfectin dramatically increased the DNA efficacy, as the administration of Ag85B DNA in saline resulted in only a threefold reduction in RLU; moreover, the latter reduction was found only during the first month after challenge. At the spleen level, only mice vaccinated with Ag85B DNA in Vaxfectin showed a weak, albeit statistically significant, reduction in mean RLU at week 4 after challenge. Ag85B DNA in saline was not protective at either time point in the spleen. Increased protective efficacy of Vaxfectin-formulated Ag85B DNA was not caused by nonspecific activation of the immune system by the adjuvant, as RLU in mice injected with Vaxfectin only were not

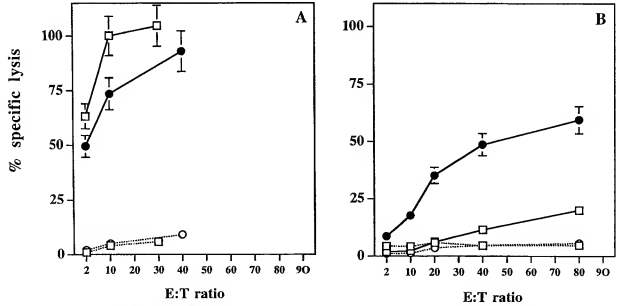

FIG. 2. CTL activity in cultures of spleen cells from B6 mice vaccinated with PstS-3 DNA in saline (open squares) or in Vaxfectin (filled circles) as measured 1 month after vaccination (A) or 5 months after vaccination (B) against ^{51}Cr-labeled RMA target cells pulsed with peptide (symbols with solid lines). Open squares and circles with broken lines represent percent lysis of RMA target cells without peptide. Data are means and standard deviations. E:T, effector to target cell.

TABLE 5. Increased protective efficacy of Ag85B DNA against intravenous *M. tuberculosis* challenge by formulation in Vaxfectin

Organ	Vaccine	Mean $\log_{10}$ mRLU/organ ± SD following *M. tuberculosis* challenge at wk indicated (no. of animals) [change]		
		2	4	8
Lungs	None	4.76 ± 0.20 (5)[a] [+0.41]	4.94 ± 0.34 (7)[a] [−0.32]	5.29 ± 0.22 (7)[a] [+0.04]
	Control DNA	4.35 ± 0.25 (5)	5.26 ± 0.14 (5)	5.25 ± 0.20 (3)
	M. bovis BCG	3.43 ± 0.28 (3)[b] [−0.92]	3.51 ± 0.02 (2)[b] [−1.75]	4.36 ± 0.46 (2)[b] [−0.91]
	Ag85B-saline	3.92 ± 0.31 (5)[b,c] [−0.43]	4.79 ± 0.18 (5)[a,d] [−0.47]	5.10 ± 0.16 (5)[a,d] [−0.15]
	Ag85B-Vaxfectin	3.49 ± 0.18 (5)[b] [−0.86]	4.28 ± 0.22 (5)[b] [−0.98]	4.45 ± 0.26 (5)[b] [−0.80]
Spleen	None	4.45 ± 0.09 (5)[e] [+0.23]	3.86 ± 0.12 (7)[e] [−0.27]	3.96 ± 0.22 (7)[e] [+0.15]
	Control DNA	4.22 ± 0.13 (5)	4.13 ± 0.19 (5)	3.81 ± 0.20 (3)
	M. bovis BCG	3.47 ± 0.06 (3)[f] [−0.75]	3.47 ± 0.02 (2)[f] [−0.66]	3.87 ± 0.58 (2)[e] [+0.06]
	Ag85B-saline	4.16 ± 0.05 (5)[e,c] [−0.06]	4.02 ± 0.28 (6)[e,c] [−0.11]	3.74 ± 0.04 (5)[e,d] [−0.07]
	Ag85B-Vaxfectin	4.16 ± 0.16 (5)[e] [−0.06]	3.77 ± 0.14 (5)[e] [−0.36]	3.64 ± 0.14 (5)[e] [−0.17]

[a] Not significant compared to results obtained with control DNA.
[b] For a comparison with control DNA, the P value was <0.005.
[c] For a comparison with Ag85B DNA in Vaxfectin, the P value was <0.025.
[d] For a comparison with control DNA, the P value was <0.05.
[e] Not significant compared to results obtained with Ag85B DNA in Vaxfectin.
[f] For a comparison with Ag85B DNA in Vaxfectin, the P value was <0.005.

significantly different from RLU in naive mice (4.03 ± 0.28 [n = 6] versus 4.01 ± 0.49 [n = 5] $\log_{10}$ mRLU for the lungs and 3.85 ± 0.09 [n = 6] versus 3.74 ± 0.16 [n = 5] $\log_{10}$ mRLU for the spleen at week 4 in a separate experiment).

DISCUSSION

Since the initial observations in the early 1990s, the scientific literature on DNA vaccination has been growing exponentially, and it is now generally accepted that this technique is a powerful means for inducing protective humoral and cell-mediated immune responses (at least in small rodents) against a number of viral, protozoal, and bacterial pathogens (7, 39). Also, for mycobacterial diseases and TB in particular, many reports have discussed the use of DNA vaccines in experimental mouse and guinea pig models (for a review, see reference 17). Among the wide range of TB DNA vaccine candidates tested so far, vaccines consisting of DNA encoding components of the Ag85 complex rank among the most promising, and reproducible protective efficacy has been reported by a number of independent research groups (1, 19, 22; I. Orme, personal communication). However, even the best TB DNA vaccine candidates actually identified need considerable improvement before clinical studies can be undertaken. Indeed, intramuscular DNA vaccination is particularly effective in priming $CD4^+$-T-cell-mediated Th1-type and $CD8^+$-T-cell-mediated CTL responses, but the small amount of actual protein synthesized in the host (nanogram amounts of protein for micrograms of administered pDNA) is a serious limitation of this type of immunization. Prime-boost strategies of consecutive DNA priming followed by boosting with protein (37) or with attenuated poxviruses (29, 30) have the potential to increase the effectiveness of these TB DNA vaccines to a certain extent.

Here we have shown that formulation of pDNA vaccines consisting of DNA encoding three *M. tuberculosis* antigens with novel cationic lipid formulations is another effective means for increasing their immunogenicity and protective efficacy. Cationic lipid formulations have been reported to enhance antibody responses induced by pDNAs given by the intranasal route (Sukhu et al., Abstr. 2nd Annu. Meet. Am. Soc. Gene Ther.) and the intramuscular route (13, 32), but little is known so far about their effect on Th1-type cytokine responses. From the results presented here, it is clear that these cationic lipids can function as strong adjuvants for $CD4^+$-T-cell-mediated IL-2 and IFN-γ production as well. Moreover, and somewhat in contradiction with previous reports (13, 32), we found that Vaxfectin also had a favorable effect on $CD8^+$-T-cell-mediated responses. Vaxfectin had no effect on CTL responses mea-

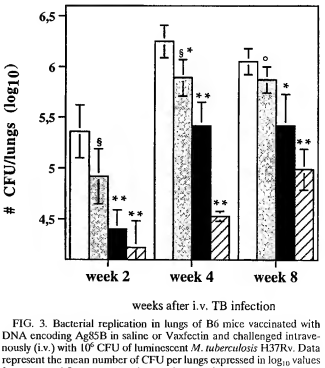

FIG. 3. Bacterial replication in lungs of B6 mice vaccinated with DNA encoding Ag85B in saline or Vaxfectin and challenged intravenously (i.v.) with 10^6 CFU of luminescent *M. tuberculosis* H37Rv. Data represent the mean number of CFU per lungs expressed in $\log_{10}$ values for groups of five to seven mice vaccinated with empty vector (white bars), with Ag85B DNA in saline (grey bars), with Ag85B DNA in Vaxfectin (black bars), or with *M. bovis* BCG (hatched bars). P values were as follows: *, $P < 0.05$; **, $P < 0.005$ (compared to value for mice vaccinated with control DNA); §, $P < 0.01$; °, $P < 0.025$ (compared to value for mice vaccinated with Ag85B DNA in Vaxfectin).

sured shortly after immunization, but CTL memory was more sustained in the Vaxfectin-treated animals. It can be speculated that this increased CTL memory was the consequence of a higher level of IL-2 production in Vaxfectin-treated animals, which might have had a direct effect on the generation of CTL memory precursors during immunization. The fact that we used higher doses of DNA and three immunizations instead of two might explain the discrepancy between our results and those of other published reports.

It is not clear at the moment how Vaxfectin exerts its adjuvant effect. The lipid does not facilitate plasmid transfection of myocytes, nor does it increase the transcription or translation of a β-galactosidase reporter plasmid in muscle tissue (13). On the other hand, Vaxfectin may increase the plasmid transfection of other cells, such as lung epithelial cells (42), macrophages, and dendritic cells (DCs). DCs are known to migrate from the tissues to the lymph nodes within 24 h when stimulated to maturity by exposure to lipopolysaccharide via Toll-like receptor 4 (TLR4) signaling. The migration and maturation of DCs may be enhanced by Vaxfectin through a similar stimulation of TLR4; moreover, this process could take place in concert with the stimulation of TLR9, which recognizes specifically immunostimulatory CpG motifs in bacterial pDNA (3, 14). Also, the adjuvant effect may involve increased antigen presentation. as cationic lipids related to Vaxfectin (specifically DMRIE:DOPE) are known to upregulate MHC class I molecules on tumor cells in tissue cultures (12). Moreover, cationic lipids certainly protect pDNA from nuclease degradation, and this effect may be of particular importance for mucosal immunizations. Finally, lipid-DNA complexes may have inherent stimulatory properties for Th1 and B cells through the induction of IL-12 or IFN-γ (8) and IL-6 (32), respectively.

Confirming previous findings with Ag85A DNA (9, 38), the protective efficacy of the DNA was only transient, and mice vaccinated with Ag85B DNA in saline were protected only during the first weeks after challenge. In contrast, mice vaccinated with the Vaxfectin DNA formulation showed a higher and more sustained reduction in CFU and RLU in the lungs. A number of factors, such as impaired signal transduction in IFN-γ-activated lung macrophages and increased secretion of suppressive factors, such as transforming growth factor β, may be involved in the waning of protection (11). It can be speculated that in DNA-Vaxfectin-immunized mice, larger numbers of Ag85-specific precursor and effector T cells might result in more strongly activated macrophages in which mycobacteria would be more efficiently eliminated, in turn resulting in a lower bacterial burden in macrophages. less impaired signal transduction, and a lower level of secretion of suppressive factors.

TB infection has been reported to specifically downregulate MHC class II expression (15); hence, Ag85-specific $CD4^+$ T cells may become ineffective at some point in time because the relevant epitopes are no longer presented on infected cells. As neither Ag85A nor Ag85B contains K^b- or D^b-restricted epitopes in its sequence (9), the totality of the immune response induced by Ag85 DNA vaccines in *H-2*^{b} mice is mediated by $CD4^+$ T cells. With the progression of infection, certain proteins of *M. tuberculosis* can escape from phagosomal containment and reach the cytoplasm, where they become accessible to MHC class I-restricted presentation (31). For Ag85 and B6 mice, this rescuing MHC class I pathway may be completely lacking. A combination of the Ag85 DNA vaccine, which stimulates strong $CD4^+$-T-cell responses, with CTL epitopes, such as the D^b-restricted epitope of the PstS-3 lipoprotein, described in this report, may help to overcome this problem (M. Romano, unpublished data). In summary, we have shown that DNA vaccines consisting of DNA encoding three *M. tuberculosis* antigens can be formulated in cationic lipids, resulting in increased antibody production and Th1-type cytokine secretion in the spleen and more importantly in the lungs, more sustained $CD8^+$-T-cell-mediated CTL memory, and prolonged protection against *M. tuberculosis* challenge. Cationic lipids can be easily manufactured and have been found safe and well tolerated in animal and clinical trials. Our results may therefore be of importance for the future clinical use of TB DNA vaccines in humans.

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